SUMMARY OF DOCTORATE THESIS

STROMAL PARTNERS INVOLVED IN ANGIOGENESIS OF ORAL SQUAMOUS CELLS CARCINOMAS: HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

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Introduction

Tumor-host interaction is a complex feature of tumor progression and an increasingly important objective of anticancer strategies. It involves interaction between cancer cells, immune effector cells and inflammatory cells and also stromal cells and tumor vasculature. Neoplastic cells are influenced by tumor microenvironment and vice versa. Angiogenesis is a complex event mediated by angiogenic factors released from cancer cells and immune cells of the host. They seem to play an important role in the interrelations of tumor cells surrounding the various cellular constituents of the stroma (tumor microenvironment), thus also contributors to induce angiogenesis indirectly. Between tumor cells and stromal tumor growth initiated a two-way relationship, which favors tumor growth.

Inflammatory events may create a local microenvironment capable of promoting tumor growth and progression. Tumor cells and/or tumor microenvironment cells, respond to hypoxia with tumor necrosis, inflammation and the release of a number of growth factors and cytokines, which are chemoattractive for monocytes and macrophages. In turn, macrophages secrete growth factor that affect tumor cells or tumor vessel endothelium and promotes the recruitment of secondary inflammatory cells such as mast cells and neutrophils. Tumor cells support the tumoral progression by sustaining the inflammatory process and secrete protumorigene and proangiogenic cytokines.

Angiogenesis in oral squamous cells carcinoma

Factors involved in angiogenesis of oral squamous cells carcinoma

Formation of "tumor-associated vasculature", is known as tumoral angiogenesis, an essential process for tumor progression. Tumor angiogenesis is the result of an imbalance between positive and negative angiogenic factors produced by both tumor cells and host.

Proangiogenic factors involved in oral squamous cells carcinoma tumoral angiogenesis

Proangiogenic acting factors are: VEGF (vascular endothelial growth factor) and angiopoietins 1 and 2. According to current nomenclature, they are recognized three types of VEGF tyrosine kinase receptors: VEGFR-1 (FLT-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (FLT-4). The VEGF acts specifically on vascular endothelial cells causing: induction of
endothelial cell proliferation and migration and potency of the microvasculature, being 50,000 times more potent than histamine. In this way it facilitates the phenomenon of neoangiogenesis in oral squamous cell carcinomas.

Stromal partners of tumoral angiogenesis

• Macrophages are frequently observed in the stroma of malignant neoplasms, and they have an important role in mediating tumor angiogenesis, in tumor growth, in stroma formation, metastasis and malignant cell degeneration. Their role in angiogenesis is complex, producing proangiogenic factors, over 20 substances that stimulate proliferation, migration and differentiation of endothelial cells. They may modulate angiogenesis by influencing extracellular matrix composition and morphology of endothelial cells and thus increase neocapillaris.

• Mast-cell seems to be a promoter of normal and tumoral angiogenesis, including oral squamous cells carcinoma. Angiogenic factors including VEGF, bFGF and platelet derived growth factor, they have been reported as stimulators of mast-cells migration. Mast cells could produce angiogenic factors that stimulate increased infiltration with mast cells

Study objectives

Oral cancer is a major health problem worldwide due to high incidence and low survival rate, as well as functional and cosmetic defects that accompany the disease and treatment. The issue raised is of great necessity arising from the introduction of therapy with antiangiogenic agents whose target is tumor microvasculature.

The main objective of the present study including those derived from here, are:

1. The analysis of the main clinical and morphological parameters of oral squamous cell carcinomas: study of clinical parameters: sex, age, topography, risk factors; the study of morphological parameters: tumor size, shape, histopathological degree of differentiation, depth and pattern of invasion, presence/absence of metastasis, invasion of surgical resection margins, tumor stage.
2. Determination of statistically significant correlations between clinical and morphological investigated parameters;
3. Characterization of the angiogenic phenotype of oral squamous cells carcinomas with varying degrees of differentiation: the identification of the vessels type and their degree of maturation intratumoral and at the front of invasion; comparative analysis of the density and distribution of the vessels intratumoral and at the invasion front;
4. Identification of the relationship between stromal cell elements and tumor neovasculature in oral squamous cells carcinoma: the analysis of macrophages distribution and density intratumoral and at the invasion front; the analysis of mast cells distribution and density intratumoral and at the invasion front; establishing statistically significant correlations between cellular elements (macrophages and mast cells) and the vascular elements at intratumoral and invasion front level;
5. Analysis of stromal cell elements involved in angiogenesis in oral squamous cell carcinoma: analysis of the relationship macrophages - VEGF/VEGFR1/VEGFR2, analysis of the relationship mast cell - VEGF/VEGFR1/VEGFR2;
6. Establishing statistically significant correlations between the stromal, cellular and vascular elements, as well as with the clinical and morphological investigated parameters;
Materials and methods

A. Investigated material

This study was performed over a period of 4 years between 2005-2008, and included a total of 372 oral squamous cells carcinomas. From a number of 372 cases with clinical diagnosis of cancer of the oral mucosa with different locations, a total of 115 cases were diagnosed on the pieces collected by biopsy, while 257 cases were diagnosed on surgical excision. Immunohistochemical techniques were performed on a sample of 60 cases.

B. Methods

The surgical excision pieces and the biopsy pieces were processed by usual histopathological techniques in the Pathology Laboratory of the Emergency County Hospital from Craiova.

The immunohistochemical technique was performed for a lot of 60 cases using the ABC method working system (streptavidin-biotin complexes method). For simple immunohistochemical reactions we used LSAB + System-HRP system (DAKO, code K0690). The development was done with DAB (diaminobenzidine), the markings being brown. For double immunohistochemical reactions and for the double histochemical and immunohistochemical reactions, we used LSAB 2 System-AP (DAKO, code K0674) and for the development we used BCIP / NBT (bromo-chloro-iodoxilfosfat and nitro-blue-tetrazolium chloride) so that in these cases marking was dark blue, or Permanent Red with red markings. We used a particular type of catalytic system for detecting peroxidazo-neoformation vessels (CD105) (CSA II Biotin Free Tyramide Signal Amplification, DAKO, code K1497)

The morphometric analysis was performed to quantify neoformation vessels and stromal elements using the "hot spot" method. We used a morphometry software cell-D + Particle Analysis type that allows scanning and determination of vascular or cellular density throughout the cross-section.

Results

A. Histopathological study of oral squamous cells carcinomas

The tumors were diagnosed in patients aged in a wide range between the IIrd and Xth decade of life. The incidence of injuries by gender, showed a clear predominance of this disease in males in which I found 299 of the 372 studied oral carcinomas. Relating to tobacco and alcohol consumption, we found the combination of these two risk factors, and also preexisting lesions of the oral mucosa. Another parameter analyzed was the location of lesions. Most of the tumors analyzed originated from the lips (46.77%).

Maximum tumor diameter could be appreciated only for 242 cases, because for the remaining cases were performed only diagnostic biopsies, resulting in pieces of 0.5-1 cm.

The tables of incidence analysis showed highly significant correlation between the location of the lip and the categories T1-T2 (p = 0.027, chi square = 4.912, for the greatest liberating 1), which exceeded the 95% confidence interval (OR = 3, 14). Fisher's exact test had a value of 0.03, signifying a positive correlation between the parameters analyzed. Lingual location highly significant correlated with T3-T4 groups (chi square = 15.85, p = 0.00), exceeding the 95% confidence level (OR = 6).
Histopathological study revealed that most of the analyzed oral squamous cells carcinomas were non-keratinized forms (197 cases, constituting half of the casuistry under consideration (52.96%).

Regarding the degree of differentiation of tumoral squamous cells, the investigated carcinoma were well differentiated, moderately differentiated and poorly differentiated.

Another parameter taken into consideration in this study was the tumor progression stage. Frankly invasive carcinoma accompanied by metastatic lymphadenopathy was present in 10 cases, representing 3.90% of the analyzed casuistry: two of the cases in an ipsilateral lymph node (N1), a case in an ipsilateral lymph node (N2a), in seven cases in multiple ipsilateral lymph nodes (N2b).

Invasion pattern was: expansive, infiltrative or presented both characters. In a number of 10 cases showed tumor masses of neoplastic emboli within blood and lymphatic vessels in 18 cases we observed aspects of neoplastic per neural and per vascular invasion.

To assess the degree of tumor progression by encasing the appropriate the patients in the appropriate stage of the disease, we used the WHO 2005 staging system. Most of the cases, respectively 140 (60.60%), were classified as stage I. We conducted a series of statistical tests to determine correlations between histopathological parameters taken in the study. By performing chi-square test, we obtained statistically significant correlations between the degree of differentiation of tumor and tumor stage, tumor size (T), and the number and size of the lymph nodes (N).

Another parameter microscopically analyzed in our study was the presence of residual malignant cells at the surgical margins of safety.

B. Immunohistochemical study of stromal cell partners in angiogenesis of oral squamous cells carcinoma
B.1. Analysis of stromal cells (macrophages and mast cells) and vascular elements intratumoral and at the invasion front
B.1.1. Analysis of the density and distribution of the blood vessels

Comparing averaged vascular microdensity quantified using CD105 intratumoral and at the invasion front, Student t-test showed a p-value = 0.0007, which confirmed a characteristic distribution of these elements in those areas.

B.1.2. Analysis of the density and distribution of the macrophages

We analyzed CD68+ macrophage density intratumoral and at the invasion front. Macrophage density was higher in the tumor invasion front than intratumoral. From the statistical point of view we found a linear correlation between the vessels and the distribution of macrophages at the invasion front (Pearson coefficient = 0.63), and the absence of such a correlation in the tumoral area (Pearson coefficient = 0.067); t-Student test showed a p-value = 0.024, which indicates the characteristic distribution of macrophages intratumoral and at the invasion front. I have not noticed any correlation between macrophage density at intratumoral and invasion front level with tumor location (chi-square, p = 0.21 and p = 0.051) or stage of tumor progression (Chi-square, p = 0.12 and p = 0.21). However, we found that intratumoral and tumor invasion front macrophage density was higher in poorly differentiated invasive tumoral areas (Chi-square, p = 0.20 and p = 0.03, significant value).
B.1.3. Analysis of the density and distribution of the mast cells

I appreciated the mast cell density through the mast cell tryptase immunoreactions and it was identified in all selected cases. The distribution of mast cells both at the tumor, but especially in the invasive areas was near the vessels or perivascular. Statistical analysis of mast cells and vessel density shows a linear correlation between mast cell density and vessels at intratumoral level and a low linear correlation at the invasion front. T- Student test of comparing the average density of mast cells intratumoral and the invasion front showed highly statistically significant value for the distribution of mast cells \( p = 3.23 \times 10^{-8} \). The same test showed a highly statistically significant value when comparing the vascular density and mast cells density at the front of tumor invasion \( (1.11 \times 10^{-8}) \). Morphologically, mast cells showed varying sizes and varying degrees of degranulation.

B.2. Analysis of stromal elements, of the vascular-endothelial growth factor (VEGF) and its receptors 1 and 2 (VEGFR1 and VEGFR2) intratumoral and at the front of invasion

Immunoreactivity for VEGF, VEGFR1 and VEGFR2 was identified in all 60 selected cases for this study, having a intratumoral variable intensity level in the cytoplasm of tumor cells.

B.2.1. VEGF immunostaining analysis

VEGF immunostaining was present in 91.6% of the analyzed cases. VEGF expression was more intense in tumor cells compared with its expression in the tumoral blood vessel endothelium.

B.2.2. VEGFR1 şi VEGFR2 immunostaining analysis

VEGFR1 immunostaining was present in 80% of the analyzed cases, while VEGFR2 immunostaining was present in 65% of them. VEGFR1 and VEGFR2 immunostaining was present both in the tumoral cells and vascular endothelium. We found no correlation between VEGFR1 and VEGFR2 expression and histopathological investigated parameters.

B.2.3. Study of VEGF and its receptors (VEGFR1 and VEGFR2) expression and distribution of mast cells and macrophage

For VEGF, and its analyzed receptors, R1 and R2, in the tumoral stroma and invasive areas, we found the positivity of some stromal elements, components of the inflammatory response or adjacent to vessels. I choose for the double immunostaining of those slides to demonstrate the immunohistochemical co-expression in stromal components, which demonstrates the stromal elements involvement in the secretion of angiogenic factors and, consequently, their involvement in tumor angiogenesis. Double immunohistochemical reactions VEGF/CD68, VEGFR1/CD68, VEGFR2/CD68 and VEGF/Tryptase, VEGFR1/Tryptase, VEGFR1R2/Tryptase showed that these cellular elements were represented mainly by macrophages and mast cells. The number of mast cells that expressed VEGFR1 and VEGFR2 was on average 18% respectively 10% of the total of these items, without a particular distribution intratumoral or at the tumor invasion front. There have been better highlighted aspects of their various degrees of degranulation.
Conclusions

1. The distribution of tumors according to age and sex groups: 32.25% of cases are diagnosed in the seventh decade of life, and it was a clear predominance in males (80.38%);
2. Risk factors for oral carcinogenesis were frequently associated with each other - especially alcoholism and smoking were found in patient’s past, in 9.33% and 16.34% respectively, and their association was present in 64.60% of analyzed cases;
3. Location of lesions: most of the tumors originated on the lips (46.77%), followed by lingual location (22.31%), the rest of localization is the floor of the mouth (14%), the hard palate (10.20%) and the gums (6, 72%);
4. The most common histopathological type of CSO was the non-keratinized form (52.96%), followed by keratinized form (42.47%), the adenoid form (1.61%), the basaloide form (1.61%), the spindle cells and warty forms (0.54%) and I found just one case of papillary carcinoma (0.27%).
5. Regarding the degree of differentiation: well differentiated forms were most numerous, that being probably due to their maximum incidence to the lips (46.77%), which corresponded to keratinized forms and warts type of squamous carcinomas. Moderately differentiated types were present in over one third of the tumors (39.51%), including non-keratinized and adenoid forms. Poorly differentiated forms included some non-keratinized, the basaloide and the spindle cells forms (18.82%) and had a maximum incidence on the tongue;
6. Regarding pTNM staging, I concluded: stage I - 60.60% of cases, stage II - 28.14% of cases, stage III - 10.82%, and stage IVA - 0.44% of cases;
7. Statistical tests for the parameters taken into histopathological study showed correlation between tumor stage and tumor location: highly significant correlation between the location on the lips and T1-T2 stage (p = 0.027), location on the tongue was high significantly correlated with -T3 T4 stage (p = 0.00), highly significant correlation between the degree of differentiation and tumor staging, p-value is close to 1;
8. The analysis of 60 cases of oral squamous cells carcinomas with varying degrees of differentiation for which we followed stromal elements (CD68, triptaza, Alcian blue-safranin) and vascular elements (CD31, CD105, VEGF, VEGFR1, VEGFR2) at both intratumoral and the invasion front, highlighted the involvement of stromal cell elements (macrophages and mast cells) in angiogenesis in the CSO;
9. Average values of MVD (CD105) intratumoral and at the invasion front has confirmed the characteristic distribution of vessels, the number of CD105 positive vessels was higher at the invasion front (p = 0.0007);
10. Comparison of the average values of macrophages density intratumoral and at the invasion front (p = 0.024), showed numerical superiority of the macrophages in areas of tumor invasion compared to the intratumoral ones;
11. I found a linear correlation between the distribution of vessels and macrophages in the front of invasion (Pearson coefficient = 0.63) and the absence of such a correlation in the tumor area (Pearson coefficient = 0.067);
12. Highlighting CD68 + monocytes in the lumen of vessels demonstrates the origin of tissue macrophages in circulating monocytes;
13. Macrophage density was higher in the invasive poorly differentiated areas (p = 0.20 and p = 0.03), suggesting that macrophages may be a predictor of tumor aggressiveness;
14. The mean mast cells density intratumoral and at the invasion front was less statistically significant for histochemical method \((p = 0.043)\) than for the immunohistochemistry \((p = 3.23 \times 10^{-8})\);

15. Mast cell density was significantly correlated with the degree of differentiation only at the front of invasion \((\chi^2\text{-square}, p = 0.04)\);

16. Mast cells density and microvessels density were statically significant correlated at intratumoral level \((\text{Pearson coefficient} = 0.47)\) and significantly weaker in the tumor invasion front \((\text{Pearson coefficient} = 0.19)\). Correlation between mast cells density and microvessels density at intratumoral level would suggest that mast cells promote tumor progression by regulating tumoral angiogenesis;

17. The distribution of the average density of macrophages and mast cells showed a highly statistically significant value at intratumoral level \((p = 0.001)\) and highly statistically significant \((p = 4.53 \times 10^{-5})\) at the tumor invasion front;

18. The distribution macrophages and mast cells showed a statistically significant linear correlation at the invasion front \((\text{Pearson coefficient} = 0.39)\) and statistically significant weak correlation at intratumoral level \((\text{Pearson coefficient} = 0.13)\);

19. VEGF and CD105 expression in tumor microvasculature is higher at the level of the invasion front, resulting in a significant difference between the angiogenesis of the tumor invasion front compared with intratumoral angiogenesis, which proves that the angiogenesis in the CSO is more pronounced at the front invasion than intratumoral;

20. Positive reactions of co-expression (Triptază, CD68 vs VEGF, VEGFR1, VEGFR2) in the stromal cells demonstrate their involvement in the secretion of angiogenic factors and consequently in the tumor angiogenesis.

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